Characterization of a Human Granulocytic Ehrlichiosis-like Agent from *Ixodes scapularis*, Ontario, Canada

To the Editor: Human granulocytic ehrlichiosis (HGE), a tick-associated febrile illness first described in Minnesota and Wisconsin in 1994 (1), has recently been reported in a number of European countries (2,3). Molecular and serologic characterization has shown that the HGE agent is closely related or identical to *Ehrlichia equi* and *E. phagocytophila* (4,5). In the United States, human cases of HGE overlap the range of the blacklegged tick, *Ixodes scapularis*, and the detection of HGE agent DNA in this species provides direct evidence that this arthropod is a competent vector (4). We report the first identification and characterization of an HGE-like agent in a blacklegged tick collected in a tick-endemic area of Canada (6).

Sixty male and 60 female *I. scapularis* were collected from five white-tailed deer shot on Long Point Peninsula, Ontario, during November 1999. Live ticks were cut longitudinally into halves, and half of each specimen was placed in lysis buffer from a QIAamp DNA Mini Kit (Qiagen Inc., Canada); DNA was extracted per manufacturer's instructions. Five microliters of extracted tick DNA was then added to a polymerase chain reaction (PCR) mixture containing primers Ehr 521 and Ehr 790 (7), and the resulting amplification products were run on agarose gels. Extracted DNA from one male tick generated the expected 293-bp HGE agent amplicon. Preliminary DNA sequencing analysis of the putative granulocytic *Ehrlichia* PCR product indicated a high sequence identity with HGE agent 16S rDNA. To further genotype this HGE-like agent, an 894-bp portion of 16S rDNA was amplified by using primers ge3a, ge9f, ge10r (8), and primer mdge9r (5' ATGTCAAGGAGTGGTAAGGT) in a nested PCR reaction.

Genetic characterization of the Long Point HGE-like agent (designated here as L3H) was carried out by sequencing an 849-bp portion of the rDNA gene and comparing it with other HGE-like agents in GenBank. Within the rDNA portion sequenced, L3H shares 99.6% identity with the HGE agent and *E. equi*/*E. phagocytophila*. In the 849-bp portion of the rDNA gene amplified and sequenced, the L3H strain differed from the HGE agent by three nucleotides. Comparison of L3H with HGE-like agents from the United States, Europe, and China suggests a high degree of sequence identity at the rDNA level; however, a number of nucleotide positions did show variation. (GenBank accession number for L3H is AF311343.)

This study documents for the first time (by rDNA sequence comparisons) that *I. scapularis* from a tick-endemic site in Canada can harbor an ehrlichia of the *E. equi* genogroup and is closely related to the HGE agent. The taxonomic significance of HGE-like agents that vary somewhat in their rDNA sequence is still unclear. HGE-like agents from diverse geographic locations and various hosts can exhibit nucleotide differences at a number of positions and still be >99% similar at the level of rDNA sequence identity. Recently it has been shown that sequencing of a more variable genomic region, such as the *ank* gene of granulocytic ehrlichia, can group these agents into different North American and European genetic clades or genogroups (9). Whether all HGE-like "variants" that differ somewhat in their rDNA or *ank* gene sequences can cause human or animal disease remains to be determined.

The identification of an HGE-like agent further highlights the concern that *I. scapularis* may transmit a number of pathogens to humans or other animals in Canada. Public health officials and veterinarians should be aware of this finding and consider HGE in the differential diagnosis of patients or clients with relevant clinical presentations. Further studies documenting the prevalence of the HGE-like agent(s) in ticks from Canada and characterization of any agents identified are warranted to better define potential human and animal health risks.

Michael A. Drebot,* Robbin Lindsay,*
Ian K. Barker,† and Harvey Artsob*
*Health Canada, Canadian Science Centre for Human and Animal Health, National Microbiology Laboratory, Winnipeg, Manitoba, Canada; and †Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada

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High Prevalence of Sin Nombre Virus in Rodent Populations, Central Utah: A Consequence of Human Disturbance?

To the Editor: Sin Nombre virus (SNV) (Bunyaviridae) is a newly discovered hantavirus responsible for hantavirus pulmonary syndrome (HPS) in humans. The deer mouse, *Peromyscus maniculatus,* is its primary reservoir (1,2). To address a gap in our understanding of the temporal dynamics of SNV, we conducted a longitudinal study in the Great Basin.

Our study site was the West Tintic Mountains, Jericho, Utah, 39°57' N, 112°22' W. We trapped on May 29 to 31, July 10 to 12, and October 7 to 9, 1999. Previous research (3) indicated woodrats (*Neotoma lepida*) were reservoirs for SNV; therefore, we concentrated our trapping efforts at woodrat middens. Middens (2 m diam) are structures of thousands of sticks built by woodrats and serving as nesting sites for a variety of small rodents, including deer mice (4,5). Each night of the 3-night trapping session, we set ~3 live traps baited with oats, peanut butter, and cotton at each of ~40 middens.

Captured rodents were collected each morning and anesthetized with Metafane (methoxyflurane). Animals were identified to species and ear-clipped for future identification. Scarring, body mass, sex, tail length, and reproductive status were recorded (data available by request). Animals were bled via the retroorbital sinus. We performed an enzyme-linked immunosorbent assay for detection of hantavirus antibody (3).

We trapped six species of rodents; *P. maniculatus* was the most common, followed by *P. truei* and *N. lepida*. Other species captured infrequently were *Dipodomys ordii*, *Largurus curtatus*, and *Chaetodipus* sp.

Over three trapping periods, we captured 212 *P. maniculatus;* 63 were antibody-positive (29.7%). Prevalence of SNV was greater in males than in females (chi-square = 3.8, p = 0.05), and it varied little among sampling periods. Most of the variation was due to changes in prevalence in males, which was 28% to 42%; prevalence among female deer mice was 17% to 20%. *P. truei* also tested positive for SNV. Of 37 *P. truei* tested, 4 were antibody-positive (10.8%).

We found a high and relatively stable level of SNV prevalence in a population of deer mice in Central Utah. Mean antibody prevalence (29.7%) across 3 periods was up to 3 times higher than that of other locations. Prevalence of SNV in this population was comparable with that during the 1993 outbreak of HPS in the Four Corners region.

We propose that the high level of SNV prevalence could be due to disturbance by humans, primarily intensive use of all-terrain vehicles at the study site. Little Sahara Recreation Area, ~4 km from the study site, recorded 180,000 visitors during 1999, mostly all-terrain vehicle users (Bureau of Land Management statistics). Many visitors to Little Sahara camp and recreate on land in our study area. This heavy recreational use has produced numerous dirt roads and campsites. Vehicle movement has denuded the area of vegetation other than large (>1 m tall) shrubs and trees and has removed cryptogamic crust, resulting in compaction of sandy soil into roads, trails, and large open spaces. Open spaces caused by disturbance reduce habitat suitable for species such as *Peromyscus* (6,7) and may cause animal density to increase within a microhabitat. Increased intra- and interspecific interactions would favor the transmission of SNV. Thus, fragmentation of the landscape may alter behavior of deer mice in a manner that enhances transmission of SNV.

Four pieces of evidence corroborate our speculation that habitat disturbance increases prevalence of SNV. First, in experimentally fragmented habitats, the density of deer mice increased dramatically. In one study, density of deer mice in small patches (4 m x 8 m) was consistently 3 times higher during 7 years of the study than that of deer mice in larger patches (10 x 50 m) (6). These small patches are similar in size to patches created by vehicle movement at our study site. Second, deer mice in fragmented habitats travel much longer distances, on average 2 times as far, as deer mice in less fragmented habitats (6). Third, immunocompetence of small rodents may decline as population density increases, making rodents more susceptible to infection than at lower densities (8). These three factors taken together should enhance transmission of SNV by increasing interactions among deer mice with lowered immunocompetence. Finally, prevalence of SNV in deer mice is lower in populations from habitats less impacted but similar to our study site. Across four other sites in the Great Basin, prevalence of SNV was 11% (9). Although we have not quantified the disturbance in these other areas, their general locations suggest they are not as disturbed by humans as the site near Little Sahara Recreational Area.

Further investigation of the effect of human disturbance on SNV prevalence is needed. We have presented several possible mechanisms that may be involved in a causal relationship between these two factors. Given that most HPS cases are contracted in areas where there has been human alteration to the landscape, future investigation of this hypothesis is warranted.

The prevalence and total numbers of infected rodents were much lower in *P. truei* than *P. maniculatus*. Adult *P. truei* are larger than *P. maniculatus* and tend to compete with *P. maniculatus* for food and nesting sites. Interspecific competition could lead to aggressive contact between these two species that could result in interspecific transmission of SNV. *P. truei* were regularly captured at the same middens on the same nights with *P. maniculatus*.

We suggest that the high level of disturbance at this study site could increase the probability of SNV transmission between species through the same mechanisms suggested for the high levels of prevalence within deer mice. Rodents at our study site may be living at higher densities than in other areas. The increased contact between species, especially when SNV prevalence is high in deer mice, could promote transmission to species other than *P. maniculatus*.

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Rachel Mackelprang,* M. Denise Dearing,* and Stephen St. Jeor†
*University of Utah, Salt Lake City, Utah, USA;
and †University of Nevada, Reno, Nevada, USA

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Mycobacterium tuberculosis Isolates of Beijing Genotype in Thailand

To the Editor: Anh and colleagues recently reported that in new tuberculosis (TB) cases from Vietnam, *Mycobacterium tuberculosis* isolates of the Beijing genotype are associated with younger age and, in isolates from Ho Chi Minh City, with resistance against isoniazid and streptomycin (1). However, occurrence of Beijing genotype *M. tuberculosis* strains may be different in other Southeast Asian countries such as Thailand.

From May 1999 to June 2000, we obtained 244 *M. tuberculosis* isolates from TB patients at Ramathibodi Hospital, Bangkok, Thailand (the hospital treats approximately 625 TB patients annually). The isolates have been prospectively analyzed by DNA-fingerprinting with the spoligotyping method (2). Drug-resistance testing and recording of patients' data (sex, age, geographic origin) were completed for 204 of 244 patients. The 204 patients originated from all six regions of Thailand, although the central region (comprising the Bangkok area) and the northeast region predominated (59% and 23.5%, respectively). Altogether 111 male and 93 female patients with a median age of 34 years (1 to 89 years) were included. Status of BCG vaccination or HIV infection was not assessed.

The Beijing genotype was found in 90 (44.1%) of the 204 isolates analyzed in detail, without significant differences regarding the respective patients' geographic origin or sex. Thus in Thailand, the frequency of the Beijing type is somewhere between the frequency in Vietnam (53%) (1) and in peninsular Malaysia (estimated at 24%) (3). Using the same age groups as Anh and colleagues, we did not find an association of Beijing genotype with young age ($p = 0.41$; chi-square test for trend). Although Beijing type isolates were more frequent among patients <25 years (18 [56%] of 32) than among those >25 years (69 [43%] of 161), this was not significant ($p = 0.13$). This association remained not significant, if only isolates from the central or the northeast region were analyzed.

Of 204 isolates, 62 (30%) showed resistance to ≥1 of 4 drugs tested (isoniazid, 8.8%; rifampicin, 6.4%; streptomycin, 19.6%; ethambutol, 4.9%). However, overall drug resistance, resistance to single drugs, and multidrug resistance were not associated with the Beijing genotype. The frequency of resistance was similar in distribution but overall lower than reported for the Ho Chi Minh City isolates (isoniazid, 24%; rifampicin, 2%; streptomycin, 31%; ethambutol, 2%) (1).

In both studies, the highest percentage of drug resistance was found for streptomycin. In our sample, this was not associated with particular spoligotypes or with geographic origin of the patient. Furthermore, streptomycin-resistant isolates were not more frequent in older age groups, although there was a nonsignificant trend ($p = 0.12$; chi-square$_{trend}$). Streptomycin is still used for standard quadritherapy in Thailand, and occurrence of resistant strains can reflect selection or transmission recently or in the past. This differs from occurrence of streptomycin resistance in countries where streptomycin is no longer used in standard therapy (4).

In the original description of the Beijing family of strains in 1995, Beijing genotype isolates were found in 7 (37%) of 19 Thai isolates (5). In a subsequent IS*6110* restriction fragment length polymorphism analysis, 80 (38%) of 211 isolates from central Thailand collected in 1994 to 1995 belonged to the Beijing family (6). Whether 90 (44%) of 204 among our recent isolates reflect a reliable increase in Beijing type transmission over the last 5 years, is difficult to state. However, the fact that no correlation of Beijing type with (young) age of the patient was observed in the previous analysis (6) supports the notion that increasing incidence of the Beijing strain in Thai cases is not due to recent transmission.

The *M. tuberculosis* population appears to be considerably more heterogeneous in Thailand than in the large urban areas of Vietnam. In our study, the three most common spoligopatterns besides Beijing, S156, S153, and S22 (according to the nomenclature of Soini et al. [7]), together comprised 20% of 244 isolates. However, the second most frequent spoligopattern, the "Vietnam genotype" (S10 according to Soini), reportedly shared by 27% of the Vietnamese isolates, was not found in our sample of Thai isolates. Only 20 different spoligopatterns were found among 499 isolates in the Vietnam study, compared to 60

among 244 isolates in our study.

Although the Beijing genotype of *M. tuberculosis* has been recognized in settings of emerging drug resistance around the world, the situation in Southeast Asian countries with a high frequency of Beijing type isolates appears to be nonuniform.

Wolfgang M. Prodinger,* Porntip Bunyaratvej,† Roongnapa Prachaktam,† and Marion Pavlic*
*University of Innsbruck, Innsbruck, Austria; and
†Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

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Jungle Yellow Fever, Rio de Janeiro

To the Editor: Yellow fever control in Brazil through vaccination campaigns began in 1937. However, cases of jungle yellow fever still occur despite the existence of a potent vaccine and immunization campaigns focused on areas endemic for the jungle form of the disease (1). Most of these cases are in men in rural areas.

In Brazil from 1980 to 1998, 376 cases of jungle yellow fever were laboratory confirmed (by virus isolation, with or without immunoglobulin [Ig]M-capture enzyme-linked immunosorbent assay [MAC-ELISA] and immunoperoxidase stain), with 216 deaths (case-fatality rate 57.4%). Most cases were from Maranhão and Goiás States, with 99 and 41 cases, respectively; Goiás, in midwestern Brazil, reported a case-fatality rate of 95%.

During 1998 to 1999, 106 cases of jungle yellow fever were confirmed, with 40 deaths (37.7%). During 1999, 75 cases were confirmed, compared with 34 cases in 1998 and a mean of 20 cases per year from 1980 to 1998 (2). In 2000, 84 cases were confirmed, with 40 deaths (47.6% case-fatality rate). During 2000, the probable site of infection for nearly all cases was in Goiás, with 53 confirmed cases and 23 deaths, suggesting epizootic circulation of the virus (2). These cases were in unvaccinated persons who became ill in their home states after traveling to endemic areas for tourism or work.

In Brazil, almost two thirds of the territory is considered an enzootic area (3). Rio de Janeiro State is not endemic for jungle yellow fever, but in January 2000, the Oswaldo Cruz Institute confirmed a case of yellow fever in a 24-year-old woman who had traveled to a national park in Goiás State on December 28, 1999, with a group of 17 persons. Yellow fever infections were also confirmed in tourists from other states who visited this park in late 1999.

The young woman became ill on January 3 with fever, headache, retroocular pain, prostration, anorexia, and nausea. She returned to Rio de Janeiro on January 5 and visited a private clinic on January 7, when a complete blood count, platelet count, urea, creatinine, liver function tests, and dengue serologic testing were performed. The patient had leukopenia (1,730 leukocytes/mm^3), 100,000 platelets/mm^3, AST 911 U/L and ALT 680 U/L, creatinine 0.90 mg/dL, urea 10 mg/dL, and normal bilirubin and protein. Anti-dengue IgM serology was negative. A blood sample was collected January 11 for yellow fever diagnosis. Reverse transcription-polymerase chain reaction (RT-PCR) test was performed on RNA extracted from the serum (4), and virus isolation was attempted on C6/36 cells, both with negative results. A MAC-ELISA test was positive for yellow fever, with a serum IgM titer 1/80,000 8 days after onset of symptoms. The patient recovered within a week. After confirmation of this case in the only person who became ill in the travel group, yellow fever IgM serologic testing was performed on the other group members, all of whom tested negative. RT-PCR and virus isolation were not attempted because the sera were taken after the viremia period.

Control measures for the *Aedes aegypti* vector were promptly taken for a radius of 300 m around the patient's home. A vaccination campaign was carried out, during which 735 neighbors were vaccinated. An epidemiologic survey was conducted in the area by using active surveillance for all symptomatic cases of fever during the period of yellow fever transmissibility. Blood samples from patients with fever were assessed for yellow fever virus and antibodies. Surveillance was intensified immediately in Rio de Janeiro State, and our laboratory examined 54 sera from patients who had traveled recently to endemic areas and who had compatible signs and symptoms (in accordance with a nationwide protocol). All these persons tested negative for yellow fever.

From January to July 2000, >16.9 million people were vaccinated against yellow fever (2); however, cases continue to occur. Unvaccinated persons who visit yellow fever-endemic areas pose a high risk of introducing jungle yellow fever cases into nonendemic areas.

Ana M.B. Filippis,* Hermann G. Schatzmayr, * Cecília Nicolai,† Mary Baran,† Marize P. Miagostovich,* Patrícia C. Sequeira, * and Rita M.R. Nogueira*
*Oswaldo Cruz Institute, Oswaldo Cruz Foundation-FIOCRUZ,

Rio de Janeiro, Brazil; and †Municipal Health Department,
Rio de Janeiro, Brazil

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Proper Nomenclature for the HGE Agent

To the Editor: In their recent article, "Antigenic variations in vector-borne pathogens," Barbour and Restrepo discuss the outer membrane protein components of *Anaplasma marginale* and related bacteria (1). Citing a reference by Zhi et al. (2), they state that *Ehrlichia granulocytophila* is the agent of human granulocytic ehrlichiosis (HGE).

The use of new names and combinations not widely recognized for genera and species lends increasing confusion to a group of bacteria already in taxonomic disarray. Several other species names have been suggested for the HGE agent since the initial description of the clinical illness caused by this agent and the in vitro technique used to isolate the agent in blood samples (3,4). Both *E. phagocytophila* and *E. equi* are genetically nearly identical to the HGE agent, and the three are probably conspecific. Thus, most scientists in the field today would support use of the name *Ehrlichia phagocytophila* to describe these bacteria.

Recent phylogenetic analyses show that *E. phagocytophila* strains align into a clade that includes *Anaplasma marginale*, the historical precedent in this grouping. Such phylogenetic analyses, which are also supported by comparative antigenic and biological studies, have resulted in a proposal for reclassification of several *Ehrlichia* spp., including *E. phagocytophila*, into the genus *Anaplasma* (5). Until a cogent reclassification based on objective criteria is firmly accepted, the creation and use of new scientific name combinations for a single bacterium yield clinical and laboratory confusion and should be avoided.

Johan S. Bakken* and J. Stephen Dumler†
*St. Mary's Duluth Clinic, Duluth, Minnesota, USA; and
†Johns Hopkins Medical Institutions, Baltimore, Maryland, USA

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Will Avilamycin Convert Ziracine into Zerocine?

To the Editor: Dr. Courvalin urges that avilamycin be prospectively banned as an antibiotic growth promoter to prevent the development of bacteria cross-resistant to the potential human-use product evernimicin (1). Elanco Animal Health, the manufacturer of avilamycin, would like to clarify the situation with respect to avilamycin and everninomicin. It should be noted that there is incomplete cross-resistance in that enterococci resistant to avilamycin exhibit only decreased susceptibility, not complete resistance, to everninomicin (2). Dr. Courvalin's recommendation has become moot, since Schering-Plough has discontinued clinical development of Ziracin, as announced in early May 2000, "because the balance between efficacy and safety did not justify further development of the product" (http://www.sch-plough.com/news/research/2000/050500.html). Thus, avilamycin actually remains in compliance with the Swann principles. In addition, the Scientific Committee on Animal Nutrition, which advises the European Union Commission, released its assessment of the potential impact from cross-resistance in late April 2000 (http://www.europa.eu.int/comm/food/fs/sc/scan/out48_en.pdf) and concluded that, although transfer of resistant bacteria–and presumably resistance genes–from animal to human bacteria is possible, the magnitude of the transfer with avilamycin resistance was not possible to predict. In part, this conclusion reflected the early developmental status of Ziracin and a few reports of clinical experience. An extensive survey of Ziracin showed that 100% of 4,208 enterococcal isolates from patients in 27 European countries were susceptible (3). Another survey of Ziracin showed that 99.5%-100% of 6,030 isolates of methicillin-resistant *Staphylococcus aureus* E, enterococci, streptococci, and pneumococci from 33 laboratories around the world were susceptible (4). Avilamycin has been used in animal production in many of the countries from which these clinical isolates originated. To fairly balance a preemptive precautionary action against a currently marketed animal use product and a human clinical candidate, the World Health Organization Global Principles recommended that such an action be initiated only when the human clinical candidate dossier is submitted for regulatory approval, to ensure that the candidate will indeed enter the marketplace. (Use of antimicrobial growth promoters that belong to classes of antimicrobial agents used or submitted for approval in humans and animals should be terminated or rapidly phased out in the absence of risk-based evaluations.) [http://www.who.int/emc/diseases/zoo/who_global_principles.html#Purpose]). This recommendation also acknowledges, in accordance with the Swann Principles, that antimicrobial agents intended for nonhuman use can be used in animal production. The modification by the pharmaceutical industry of older classes of antimicrobials for human clinical use, with counterparts previously developed by animal health companies for use as growth promoters, has become common. Dr. Aarestrup of the Danish Veterinary Laboratory commented that "it will be necessary in the future to either totally avoid the use of antimicrobials for growth promotion or, once antimicrobials have been approved for growth promotion, to reserve these classes for growth promotion and search for therapeutic options among other classes" (2). With respect to avilamycin, this latter option is the better one, now that everninomicin (and perhaps the entire orthosomycin class by extension) has been demonstrated to be unsafe for parenteral or injectable use in humans, because it allows animal producers to use a product that poses no resistance threat to public health. Finally, other unique antibiotics for treatment of serious gram-positive infections in humans (with no animal use counterparts) are in the pharmaceutical pipeline (e.g., LY333328 and daptomycin) or have recently been approved (e.g., linezolid). We hope that a fair balance can be achieved by the human medical and the animal health and production communities with regard to the types of antimicrobial agents that can be used in each sector.

Thomas R. Shryock
Elanco Animal Health, Greenfield, Indiana, USA

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The Antibiotic Food-Chain Gang

To the Editor: In his reply to my letter (1), Dr. Shryock states that use of the growth promoter avilamycin, which confers cross-resistance to other members of the everninomycin class of drugs, was in compliance with the Swann principles. The Swann report, issued in 1969, recommends that antibiotics used to treat infections in humans not be used as animal-food additives (2). The combined efforts of many scientists were needed to bring about the 1999 ban in Europe of spiramycin, tylosin, virginiamycin, and bacitracin, each of which confers resistance to antibiotics used in clinical settings. It appears that more than 30 years was necessary for the animal-food industry to act in accordance with the Swann report.

The reasoning in terms of drug structures can be misleading. The implication is that drugs that are chemically closely related have the same target of action and are therefore subject to cross-resistance, and vice versa. For example, because it has an unusual structure, apramycin (a 4-substituted-2-deoxystreptamin) was used exclusively in animals in the hope that it would not be recognized by any of the known aminoglycoside-modifying enzymes (3). However, enterobacteria of animal origin were resistant to apramycin by synthesis of a plasmid-mediated 3-*N*-aminoglycoside acetyltransferase type IV, which also confers resistance to gentamicin (4). Following spread in animal

strains (5), the plasmid was later found in clinical isolates from hospitalized patients (6).

The use of antibiotics in general should be based on the mechanisms of resistance in bacteria, rather than on their chemical makeup. In particular, the concept that resistance was a class phenomenon rapidly lost favor because of the extension of the concept of cross-resistance and the increased occurrence of co-resistance.

In classical cross-resistance, a single biochemical mechanism confers resistance to a single class of drugs: use of a given antibiotic can select resistance to other members of the group but not to drugs belonging to other classes. However, cross-resistance between drug classes can occur by two mechanisms: overlapping targets and drug efflux. An example of target overlap is provided by the macrolides, lincosamides, and streptogramins (MLS), which are chemically distantly related. However, constitutive methylation of a single adenine residue in ribosomal RNA confers high-level resistance to the three classes of antibiotics. This resistance phenotype is due to the fact that all these antibiotics have overlapping targets on the ribosome (7). Active efflux of the drugs outside bacteria has recently been recognized as a common resistance mechanism (8,9). This energy-dependent export confers low-level resistance to a wide variety of antibiotics. The broad substrate specificities of the pumps account for decreased susceptibility to beta-lactams, aminoglycosides, tetracyclines, chloramphenicol, trimethoprim, sulfonamides, fluoroquinolones, and MLS, among others (9).

In contrast to cross-resistance, co-resistance is due to the presence in the same host of several mechanisms, each conferring resistance to a given class of drugs. In addition, the corresponding genes are often adjacent (physically linked) and expressed in a coordinated fashion. One of the most efficient system of this type is represented by the integrons (10) first described in gram-negative bacilli (11,12) and more recently found in gram-positive bacteria (13). Because of the genetic organization resulting in co-expression of the various genes, use of any antibiotic that is a substrate for one of the resistance mechanism will co-select for resistance to the others and thus for maintenance of the entire gene set. Since cross-resistance means cross-selection and co-resistance implies co-selection, the use of any antimicrobial agent is de facto rendered inadequate as a growth promoter.

I also disagree with the notion that because a member of an antibiotic class has been misused as a growth promoter the class should not be used in the future for human therapy; the hierarchy could conceivably be humans first, animals second, rather than the opposite. For various reasons, the development of daptomycin and ramoplanin has been suspended for several years. If, during this period, these agents had been used as growth promoters, they would not now be under development for humans. I would rather see ramoplanin used for the microbial modulation of the intestinal tract in immunocompromised patients than as an animal-food additive.

During the last 30 years, thanks to molecular biology, enormous progress has been made in understanding the genetics and biochemistry of resistance. Incorporating this knowledge for decision-making in problems of public health importance is timely. I hope that it will not take 30 years for the pharmaceutical industry to act in agreement.

Patrice Courvalin
Institut Pasteur, Paris, France

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Single Nucleotide Polymorphisms in *Mycobacterium tuberculosis* Structural Genes

To the Editor: A recent article by Fraser et al. (1) discussed the frequency of single nucleotide polymorphisms (SNPs) in two genomes of *Mycobacterium tuberculosis*, strains H37Rv (2) and CDC1551 (unpublished). The article contains an inaccurate representation of our published *M. tuberculosis* data on SNP frequency. The authors state that "detailed comparison of strains H37Rv and CDC1551 indicates a higher frequency of polymorphism, approximately 1 in 3,000 bp, with approximately half the polymorphism [sic] occurring in the intergenic regions. In other words, 50% of the polymorphisms are in 10% of the genome. While this rate is higher than that suggested (3), it still represents a lower nucleotide diversity than found in limited comparisons from other pathogens."

On the basis of comparative sequence analysis of eight *M. tuberculosis* structural gene loci (open reading frames [orf]), we initially published an estimated average number of synonymous substitutions per synonymous site (K_s value) that indicated that this pathogen had, on average, approximately 1 synonymous difference per 10,000 synonymous sites (4). This finding was unexpected given the relatively large population size of *M. tuberculosis* and paleopathologic evidence suggesting its presence in humans as early as 3700 B.C. Subsequent sequence analysis of two megabases in 26 structural genes or loci in strains recovered globally confirmed the striking reduction of silent (synonymous) nucleotide substitutions compared with other human bacterial pathogens (3). A large study (approximately 2 Mb of comparative sequence data) of 12 genes potentially involved in ethambutol resistance (5) and 24 genes encoding protein targets of the host immune system (6) provided data consistent with the original estimate of 1 synonymous nucleotide change per 10,000 synonymous sites in structural genes in this pathogen. Our estimate did not include SNPs located in putative regulatory regions of structural genes (intergenic regions), nor did it include nonsynonymous nucleotide changes in structural genes. These classes of polymorphisms were not included in our estimates because of difficulties in ruling out the possibility that they arose as a consequence of selective pressure due to antimicrobial agent treatment or perhaps extensive in vitro passage. Synonymous nucleotide changes (neutral mutations) are commonly used to estimate many values of interest to evolutionary biologists and population geneticists.

The estimate provided by Fraser et al. is based on a genomewide frequency of SNPs (1/3,000 nucleotide sites), 50% of which presumably are located in intergenic regions and 50% in structural genes. On the basis of a genome size of roughly 4.4 Mb, there would be roughly 1,500 total SNPs, with approximately 750 in orfs (90% of genome = 3,960,000 bp) and 750 in intergenic regions (10% of genome = 440,000 bp). On the basis of these estimates, the frequency of all SNPs located in structural genes would be roughly 1/5,280 bp. (An estimate of 1,300 total SNPs [translating to 1/6,000 bp] was presented by the group at a meeting held at the Banbury Center last December.) As expected, these numbers differ from our estimate (1/10,000), in part because they contain both synonymous and nonsynonymous nucleotide polymorphisms.

We analyzed orfs dispersed around the chromosome of *M. tuberculosis* strains CDC1551 and H37Rv and available in public databases. Surprisingly, the number of nonsynonymous SNPs exceeded the number of synonymous SNPs, yielding a synonymous SNP frequency of roughly 1/12,260 bp in orfs.

M. tuberculosis, a pathogen that infects one third of humans, clearly has an unusual if not unique molecular evolution history. Precise data on the frequency of its true SNPs genomewide are critical. At this point, data (3-6) are consistent with our original estimate of 1 synonymous nucleotide change per 10,000 synonymous sites in structural genes in natural populations of this pathogen.

James M. Musser
National Institutes of Health, Hamilton, Montana

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Response to Dr. Musser

To the Editor: In his letter on single nucleotide polymorphisms in *Mycobacterium tuberculosis*, Dr. Musser indicates that genome strain CDC1551 has not been published. Cole et al. (1) described some of the biology of *M. tuberculosis* based on the genome sequence data. The actual sequence, while not published, is in GenBank (Accession NC00962), the sequence data are available at www.sanger.ac.uk, and the annotation is available at http://genolist.pasteur.fr/TubercuList/ . We have a manuscript in preparation using a method of whole genome comparison (2) to evaluate the sequence diversity of strains H37Rv and CDC1551 and applying the information to the analysis of >150 clinical isolates. The complete sequence data and annotation for strain CDC1551 have been available for over a year at www.tigr.org and www.tigr.org/CMR, and periodic updates are provided. In addition, we are preparing to submit the strain CDC1551 sequence and annotation to GenBank (Accession AE000516).

We agree that sequencing accuracy in assessing comparative single nucleotide polymorphism (SNP) data is important. The error frequency suggested by Dr. Weinstock

("Error frequency in a finished sequence has never been precisely measured but is thought to be one error [frameshift or base substitution] in 10^3 to 10^5 bases" [3]) is not supported by any evidence. The whole-genome shotgun sequencing method developed by The Institute for Genomic Research (TIGR) (4) and adopted by many others is highly accurate because of the following qualities: 1) high redundancy in shotgun sequencing (average 7.9-fold for the strain CDC1551 project with a minimum of 2-fold coverage for any nucleotide); 2) assignment of quality values to each nucleotide base; 3) adoption of assembly programs that use quality values for consensus building; and 4) manual editing of electropherograms as necessary.

These methods were applied to the *M. tuberculosis* genome sequencing project. In comparing the CDC1551 and H37Rv strains, it is reasonable to suspect that the SNPs also have the potential to be results of sequencing errors. The sequence differences were verified by two independent methods. One hundred SNPs were chosen at random, and the base calls were independently verified by inspection of the original electropherograms at TIGR (CDC1551) and the Sanger Center (H37Rv). A second method, independent of sequencing, was also used to confirm the base calls of these 100 SNPs. The visual inspection of the electropherograms and the sequencing independent method were in good agreement and indicated that 80 (91%) of 88 successful assays of the nucleotide differences were genuine.

Since our initial report, we have improved our methods for overlaying the annotation of open reading frame coordinates onto our analysis of the coordinates of nucleotide substitutions. Approximately 7% of the genome is noncoding, and approximately 15% of the substitutions are in these regions.

Dr. Musser is correct in pointing out that the substitution frequency expressed in Fraser et al. (5), based on our preliminary annotation of our *M. tuberculosis* sequence data, is not an equivalent comparison to the synonymous substitution frequency derived by his method of sequencing a select set of genes over a wide range of *M. tuberculosis* strains. He uses the methods of Li et al. (6), among the most widely accepted, for the calculation of nucleotide substitution frequencies and derives a D_s value of <0.01 synonymous substitutions per 100 synonymous sites. Our preliminary data presented the frequency of total nucleotide substitutions at all positions (coding [synonymous and nonsynonymous] and noncoding) of the two recently sequenced strains, H37Rv and CDC1551. Our manuscript in preparation comparing the two *M. tuberculosis* strains will contain an analysis of synonymous substitutions. However, while Dr. Musser compared a select group of genes over perhaps several hundred strains, our frequency will be based on a genome-wide comparison between two strains.

Robert Fleischmann
The Institute for Genomic Research
Rockville, Maryland, USA

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